Review Article

Review of the toxicological effects of silver nanomaterials on the model aquatic organism *Danio Rerio*

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Abstract

Silver nanoparticles (Ag-NPs) are being increasingly used in consumer products, and many of the products, socks for example, are resulting in increases in environmental exposure. Zebrafish make an excellent model for aquatic ecotoxicology due to their small size, low costs, large production of transparent embryos, and extensive available tools. This review examines the potential sources of Ag-NP exposure, their fate in environmental waters and inside of the zebrafish, and potential targets and mechanisms of toxicity. With the gills being a major target illustrated by several studies, it can be hypothesized that this will be a common exposure point in aquatic species, which can lead to mortality. With the large variation in synthesis methods, and many factors influencing toxicity, it is challenging to generalize Ag-NP toxicity, which further rationalizes why an inexpensive, yet useful screening tool could greatly enhance our understanding of the implications of environmental exposure.

Introduction

Nanoparticles are materials defined as having at least one dimension less than 100 nm [1,2]. Due to many desirable properties, metal and metal oxide nanomaterials comprise one of the largest avenues for nano-engineering [3]. Soluble forms of many of these metals are toxic to aquatic life, especially silver [3]. Silver nanoparticles (Ag-NPs) are the most utilized nanomaterial and have received a great deal of attention due to their many exciting properties [4]. They have unique physicochemical properties compared to the bulk metals from which they are derived allowing for many advances in many fields [2]. Nano-sized products have been developed in the fields of drug delivery, diagnostics, tissue engineering, agricultural sciences, and environmental remediation [2,5]. As of 2013 there were a reported 1015 consumer products made from nanomaterials, with 259 of them containing nanosilver [2]. The current production estimate of Ag-NPs in the United States alone is 2.8-20 tons per annum [6]. Ag-NP concentrations in environmental waters is largely unknown, but there is a common belief among scientists that through incidental release or manufacturing discharge, that there is an impact of environmental Ag-NPs on aquatic ecosystems [6]. The broad-spectrum antibacterial activity of Ag-NPs has been exploited in many commercial products such as shampoo, food packaging, textiles, water filters, household appliances, and medical devices, all which could contribute to the release of Ag-NPs into environmental waters through use and/or manufacturing [1,2]. Due to their relatively low cost to synthesize, and mild toxicity to human cells, consumer products are being developed and utilized globally without much thought of long-term environmental effects [2]. The increased use of engineered nanomaterials has increased the exposure to humans and the environment, making it now even more pertinent to understand the potential implications of synthesis and use of Ag-NPs [7]. However, there are many contradictions in the toxicological data for these nanoparticles since there are a variety of synthesis methods [8]. The regulation of the use of Ag-NPs in consumer products must be assessed more thoroughly due to its direct impact on humans, and since they are now being used in commercial socks, which have been shown to release silver ions (Ag^+) into the water, the use of

these products may also impact environmental organisms and aquatic ecosystems [9].

Cell culture experiments have attempted to ascertain many of the toxicological properties of Ag-NPs, but cell culture experiments can be misleading. Cell culture lacks physiological barriers and intercellular communication, both of which could impact toxicity [10,11]. The pharmacokentics and pharmacodynamics of unique sizes, shapes, and coatings of Ag-NPs cannot be studied in a monoculture of cells, and therefore it is important to screen Ag-NP toxicity in animal models [10,11]. The low cost, small size, clear embryo, and available resources of the zebrafish, Danio rerio, make it a good model for toxicological assessment and make it an excellent model organism for measuring environmental fate and toxicity of Ag-NPs [11]. Zebrafish have 80% sequence homology to humans and share many common biological pathways and biological systems to mammals [12]. Exposure of Ag-NPs to zebrafish embryos results in alterations in gene expression, impacts on oxidative phosphorylation, and overall survivability of the embryos, whereas exposure to adult zebrafish influences global transcriptome profiles, endoplasmic reticulum stress, and major biochemical pathways [12]. The zebrafish embryo is one of the most widely used models of developmental biology since it develops outside of the mother and is transparent, making real-time detection of toxicological changes possible [13,14]. The short breeding cycle, low cost, and large brood size allow for hundreds of embryos to be rapidly produced making them an ideal high-throughput assay system, especially since all biological systems can be simultaneously studied [13]. It is also important to study toxic materials in adults since they are the primary source of

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bioaccumulation in an ecosystem, thus making an excellent low-cost model of ecotoxicology [8,12].

Fate of silver nanoparticles in environmental waters

Although there are many promising futures with the development of Ag-NPs, there are also environmental health concerns [15]. There is currently an estimated 500 tons per year of Ag-NPs and Ag-NP-containing products produced per annum, with little information available on the environmental release, fate, bioavailability, and toxicity to the aquatic ecosystem [2,9,15]. The use of Ag-NPs in textiles will likely increase the release of silver ions into environmental surface waters [7,12]. The approximate concentration of silver in rivers is estimated to be between 0.01 and 100 nanograms per liter, but much of that silver is complexed to negatively charged ligands found throughout surface waters [16], but increased use of Ag-NP-containing consumer products may dramatically increase those levels. Aquatic organisms are of major concern since the environmental water supply is often a sink for anthropogenic activities, including the manufacturing of Ag-NPs [4]. Ag-NPs are highly reactive due to their large surface area, and silver ions (Ag^+) have been shown to release and bioaccumulate impacting aquatic flora, specifically effecting the ion transporters of the gills of fish [2,12]. Salinity, pH, and organic matter content and composition have been demonstrated to be key factors influencing the toxicity of silver in surface waters and dissociated Ag^+ is 3-4 times more toxic than Ag-NPs in the aquatic environment [2,6,16]. Some studies suggest that freshwater fish are more highly impacted by Ag-NP toxicity since it has been shown that there is greater bioavailability of the Ag-NPs and Ag^+ in freshwater than in marine water, thus increasing the toxicity [15], although others have demonstrated that the high organic content of many freshwater systems lowers bioavailability and saline waters have higher bioavailability, implicating that marine waters would have greater toxicity from Ag^+ [2]. In addition, it has been shown that the presence of plants in the water with Ag-NPs results in decreased toxicity to aquatic organisms, such as zebrafish, most likely due to the organic exudate [6].

The Ag-NPs are thought to associate with the gills of fish and cause respiratory failure [16,17]. The release of Ag^+ from the Ag-NPs into the water decreases the available oxygen in the water creating somewhat anoxic conditions for the aquatic life [15]. Some studies indicate that Ag-NPs are acutely lethal to adult zebrafish, while others do not observe any mortality in 24-hour periods suggesting that water chemistry may play a role in Ag-NP uptake and toxicity [1,3,4,11]. In addition, there is a lot of variability in the size, coating, and synthesis of Ag-NPs, which directly impact the rate of agglomeration, uptake, bioavailability and bioaccumulation, and overall toxicity of the particles [2,8]. A current challenge of ecotoxicology is understanding what compounds in the water may interact with the Ag-NPs. The ionic strength and particle composition of environmental waters will impact the dissolution or agglomeration, and thus toxicity of Ag-NPs [8]. Chemical transformations of Ag-NPs have been widely demonstrated to impact toxicity; for example the sulfidation of the nanoparticles greatly reduces their toxicity, whereas the attachment of negative ligands can increase retention time in the biological system increasing toxicological response [12,14]. The presence of common negatively-charged ligands, such as SO_4^{2-}, S^{2-}, Cl^-, and PO_4^{3-} could easily interact with dissolved Ag^+, and the overall charge of the resulting silver greatly impacts its retention time, and thus toxicity, in the organism [12]. Since there is a wide-variety of synthesis methods currently available for the development of Ag-NPs, all with different surface coatings and rates of Ag^+ dissolution, it is challenging to predict the overall environmental impact since there

are many unique properties of nanomaterials that can impact overall toxicological effects [2].

Zebrafish exposure

Most researchers are in agreement that the gills are the primary site of exposure for Ag-NP [3], where the gills are directly affected upon exposure due to an inhibition of sodium-potassium ATPase activity resulting in a disruption of osmotic balance [2,4,15,16]. Accumulation of Ag-NPs in the gills can lead to oxidative stress, and high doses of Ag-NPs have been linked to blood acidosis and circulatory collapse, whereas lower doses strictly interact with the gill ion transporters effecting the sodium-potassium ATPase and overall ion flux [2,4,7]. Acute exposure to Ag-NPs decline the plasma electrolyte levels [sodium and chloride ions] and the potassium levels were also decreased following prolonged exposure [21 days] [4]. Plasma glucose and cortisol were also affected by Ag-NPs, with significant increases observed following both acute and prolonged exposure [4]. Cortisol is released during stress and is a reliable primary biomarker for stress, thereby suggesting that Ag-NP exposure to zebrafish results in a stressing of the animal [4]. Cortisol increases energy availability during stress, primarily through the process of gluconeogenesis, and therefore increased plasma glucose further reiterates that the fish were indeed stressed following Ag-NP exposure [4].

The Ag-NPs are also believed to be ingested by the fish resulting in oxidative stress in the intestines and accumulation in the liver [7]. There has been an observed depression in metabolic activity of zebrafish indicating that in addition to the increased production of reactive oxygen species [ROS], there may also be endoplasmic reticulum [ER] dysfunction in the livers of the fish [7]. Through ingestion or inhalation the Ag-NPs are also ending up in the bloodstream of the fish and can transverse to other tissues, including the brain, heart, and kidneys, and are impacting erythrocyte acetylcholinesterase [AChE] and blood electrolyte levels [4].

Studies have demonstrated that even sub-lethal exposure to Ag-NPs can have a profound effect on gene regulation [17,18]. A study by Griffitt, *et al.* in 2009 showed through microarrays that exposure to 26.6 ± 8 nm Ag-NPs significantly changed the gene expression patterns in the gills for 148 genes after 24 hours and 462 genes after 48 hours, most of which were suppressed [3]. Since this study concluded that there was significantly increased "whole-body silver content" following Ag-NP exposure [3], it can be hypothesized that there were many other genes impacted in the various organs of the fish. It is hypothesized that the altered physiology observed in zebrafish is due to the release of Ag^+ ions, but still others argue that there are differential effects with the Ag-NPs compared to dissolved silver due to preferential endocytosis of larger particles [10].

Hepatotoxicity

The liver is the major organ for chemical detoxification, thus it is often a target for toxicity by engineered materials [12]. Cultured zebrafish liver cells [ZFL] have been utilized to study the effects of Ag-NPs on hepatotoxicity [7]. Exposure of these cells to larger Ag-NPs [120 nm] leads to the induction of ROS, the ER stress response, and production of the pro-inflammatory cytokine TNF-α [7]. In addition to the production of ER stress response genes, the transcription of the pro-apoptotic genes p53 and Bax were also significantly upregulated following ZFL exposure to Ag-NPs suggesting induction of the apoptotic response [7]. The ER stress response occurs when there is an increase in unfolded or misfolded proteins, which activates the

unfolded protein response [UPR] leading to a suppression in protein synthesis [7]. UPR also leads to an increase in protein degradation, the induction of apoptosis, and the activation of the transcription factor NFkB, which can initiate the inflammatory cascade [7]. The ER stress response is characterized by an upregulation of the chaperone protein BiP, splicing of the X-box binding protein 1, and phosphorylation of eIF2-a, and has a substantial role in liver pathology observed after Ag-NP exposure [7]. However, when this same study was performed in zebrafish embryos instead of transformed cells, the marker for ER stress [BiP], the pro-apoptotic gene p21, and the marker for oxidative stress [Cat] were largely unchanged, suggesting that either the Ag-NPs not getting to the embryonic liver, or that the in vitro cell culture model may not be fully representative of the in vivo effects, which has been observed in many nanotoxicology studies [7].

The livers of acutely-exposed adult zebrafish are susceptible to hepatotoxicity and it is hypothesized to be linked to oxidative stress and apoptosis in the hepatocytes [1,12]. Hepatotoxicity of zebrafish is indicated by extensive cell death due to necrosis, and degenerative changes, as well as a significant increase in nitric oxide levels, which lead to oxidative stress [12]. Levels of malondialdehyde, which is a byproduct of lipid peroxidation, was observed to be increased in adult zebrafish exposed to 5-20 nm Ag-NPs indicating tissue damage [1]. In addition, glutathione production was increased in these fish which would aid in the protection against lipid peroxidation, again confirming that oxidative damage is occurring [1]. A decline in antioxidant enzymes, which normally protect against oxidative stress, was also observed following exposure to Ag-NPs, which will further augment the stress-related damage [12].

Along with the oxidative stress, an increase in DNA damage as determined by the double-stranded break marker γ-H2AX, the production of the tumor suppressor gene p53, and the upregulation of several pro-apoptotic genes [Bax, Noxa, and p21] was also occurring in the liver of Ag-NP-exposed zebrafish [1]. The metal-sensitive metallthionein 2 [MT2] gene was upregulated by these 5-20 nm Ag-NPs, thus suggesting that it is the metal ions and not the nanoparticles themselves might be causing the toxicity [1,12]. That being said, the use of larger Ag-NPs [85 nm] did not demonstrate a similar hepatotoxic effect, and total protein levels in the plasma were unaltered following acute Ag-NP exposure, thereby confirming liver health [4]. This once again reiterates the importance of understanding the impact of size and composition of individual Ag-NP batches on toxicity.

Renal toxicity

A direct inoculation into the dorsal muscle of zebrafish to examine biolocalization demonstrated that Ag-NPs can disseminate throughout the body and Ag+ can accumulate in all major organs, with highest loads observed in the kidney [11]. Induction of the inflammatory genes interleukin-1beta [IL-1β] and tumor necrosis factor alpha [TNF-α], and pro-apoptotic caspase genes were observed in the kidneys of exposed fish using quantitative reverse transcriptase polymerase chain reaction [qRT-PCR] following a 24-hour exposure of 10 nm PVP-coated Ag-NPs [11]. The fish did not die in the 24-hour period due to these cellular changes, and the study was not followed up to determine if longer exposure, or chronic exposure, would cause mortality [11].

Cardiotoxicity

Following a direct intramuscular exposure, 10 nm Ag-NPs were capable of localizing to the heart, which was the only organ examined in the study were intact Ag-NPs were found [11]. A high dose exposure

[5 mg/kg of body weight] resulted in the induction of the pro-inflammatory gene IL-1β and pro-apoptotic caspase genes in the heart tissue as determined through qRT-PCR, but in a lower dose [1 mg/kg], no significant changes were observed [11].

Developmental toxicity

It has been demonstrated in many studies that exposure to Ag-NPs can have deleterious effects on developing embryos [5,19]. The Ag-NPs can enter the embryos in less than 2 hours and interact with a variety of body systems [13,14]. Many studies have demonstrated a size-, charge-, and dose-dependent increase in mortality to embryos following exposure to Ag-NPs, with the early developmental stages being more at risk, and the surviving embryos often having malformations [5,10,13,14,20]. Smaller sizes and more negatively charged particles are more apt to cause developmental toxicity, illustrating the need for whole-animal systems to assess the pharmacokinetics and pharmacodynamics of these widely-varied particles [5,14]. Since it has been shown that exposure of female zebrafish to 10 nm Ag-NPs can result in the passage of Ag+into the eggs [11], it is important to discern the potential ramifications of long-term Ag-NP exposure to aquatic systems.

Mortality and delayed hatching are of the largest observed effects to embryos that have been exposed to Ag-NPs [9,10,21]. However, there are also numerous morphological malformations that can occur in the surviving embryos, such as a bent notochord, cardiac arrhythmia, slow blood flow, eye and tail malformations, or pericardial edema [9,10,13,21]. These effects have been linked to increased levels of oxidative stress in the tissues following acute exposure to Ag-NPs [9], and it has been demonstrated that the Ag-NPs can disseminate throughout the embryos with uniform distribution throughout the developing tissues [21].

Next-generation sequencing (NGS) analysis of the zebrafish embryo transcriptome indicated that protein synthesis and oxidative phosphorylation were the most impacted pathways following Ag-NP exposure [15]. Since the NGS study compared Ag-NPs to ionic silver and similar pathways were affected but more pronounced in the ions-exposed fish, it is hypothesized that the impacts on these pathways from Ag-NP exposure is largely due to the dissociation of Ag+ from the intact particles [15]. Although there was no mortality observed with the 10 nm Ag-NPs used in the study, the NGS transcriptomics demonstrated that there were many organs impacted and the nanoparticle exposure caused changes in the regulation of many biological processes: induction of oxidative damage, production of free radical scavenging enzymes, regulation of apoptosis, and dysregulation of cellular mechanisms for storing, detoxifying, and metabolizing metals [15].

The developing brain is a common target for heavy metal toxicity, and Ag-NPs have been shown to cause developmental neurotoxicity [10]. Ag+ and Ag-NPs have been shown to impair neurotransmitter function, and can impact neuronal cell viability [10]. Zebrafish larvae have abnormal swim behavior and became either hyporesponsive or hyperresponsive to light through exposure to smaller (10 nm) and larger (50 nm) PVP-coated Ag-NPs, respectively, but yet remained unchanged from control when exposed to 10 nm citrate-coated Ag-NPs [10].

Neurotoxicity

Due to their small size, Ag-NPs can cross the blood-brain barrier and impact neuronal function [12]. 85 nm Ag-NPs were implemented in disruption of neuroregulation following acute and prolonged exposures [4]. Erythrocyte AChE levels have been shown to

correlate with neuromuscular AChE activity [4]. Acute and prolonged studies examining the exposure of adult zebrafish to Ag-NPs have demonstrated a significant reduction in AChE activity in the blood erythrocytes, suggesting that the neuromuscular junctions of the fish are likely impaired as well following Ag-NP exposure [4]. A similar study actually examined AChE in the adult zebrafish brain, and too saw a reduction in activity [12]. AChE is involved in the breakdown of the neurotransmitter acetylcholine (Ach), and thus terminates the message from the neuron to the muscle preventing muscle contraction [22]. Inactivation of this key enzyme leads to disrupted neurotransmission with prolonged motor neuron activity, and thus continuous muscle stimulation [12,22]. This altered activity will dramatically impact animal responses and behavior following exposure [12].

Conclusion

Ag-NPs vary tremendously in their toxicological properties, mostly due to extensive variations in size, charge, coating, agglomeration, and dissolution of Ag+. There is also an environmental impact due to differences in water chemistry and organic matter content. However, this review has summarized the findings from many studies, which all indicate that Ag-NPs can adversely impact aquatic organisms. Utilizing zebrafish as a model organism, it is apparent that Ag-NPs and Ag+ can be inhaled through the gills, causing direct toxicity to them, or ingested into the organism causing wide-spread organ damage, mostly due to oxidative stress. Various studies have implicated most of the major organs as sites of Ag-NP toxicity and have shown that most sizes of Ag-NPs are capable of dissemination through the fish, without proper clearance through the kidneys.

In addition to the impacts on adult fish, it has been summarized here that the developing embryos of zebrafish experience very detrimental impacts following Ag-NP exposure, causing mortalities or malformations in most cases. Since at least the Ag+ are capable of passing from the adult fish to the egg, and perhaps even smaller sized Ag-NPs, these detrimental effects are going to impact the survivability of the species if environmental Ag-NPs increase.

Most toxicological data for Ag-NPs is based on human exposure scenarios, and much less literature is devoted to ecotoxicology. However, with the low cost, high fecundity, and ease of use of zebrafish, they do provide not only a good high-throughput model for environmental research, but also overall bioaccumulation and biolocalization studies.

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